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<b>(21) International Application Number:</b> PCT/US96/19181 <b>(22) International Filing Date:</b> 2 December 1996 (02.12.96)  <b>(30) Priority Data:</b> 08/565,856 1 December 1995 (01.12.95) US  <b>(71) Applicants:</b> UNIVERSITY OF MASSACHUSETTS [US/US]; Suite 800, 18 Tremont Street, Boston, MA 02108 (US). BETH ISRAEL HOSPITAL [US/US]; 330 Brookline Avenue, Boston, MA 02115 (US).  <b>(72) Inventors:</b> STEELE, Alan, W.; 15 Clifton Road, Wellesley, MA 02181 (US). STROM, Terry, B.; 22 Kenard Road, Brookline, MA 02146 (US).  <b>(74) Agent:</b> FASSE, J., Peter, Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110 (US).		<b>(81) Designated States:</b> AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IL-12 P40 SUBUNIT FUSION POLYPEPTIDES AND USES THEREOF  <b>(57) Abstract</b>  Disclosed are fusion polypeptides that include an IL-12 p40 subunit polypeptide covalently linked to an enzymatically inactive polypeptide. The fusion polypeptides have an increased <i>in vivo</i> half-life relative to the native IL-12 p40 subunit. The fusion polypeptides function as antagonists of the IL-12 receptor, and can be used, for example, as immunosuppressive agents (e.g., in treating autoimmune diseases or in inhibiting graft rejection) or to treat or prevent endotoxin-induced shock.		

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IL-12 P40 SUBUNIT FUSION POLYPEPTIDES AND USES THEREOFBackground of the Invention

This invention relates to fusion polypeptides that  
5 include the p40 subunit of interleukin-12 (IL-12) and an  
enzymatically inactive polypeptide.

IL-12 is a 75 kDa heterodimeric cytokine that has  
several functions *in vivo*. For example, IL-12 stimulates  
proliferation of activated T and NK cells. This cytokine  
10 also induces production of interferon (IFN)- $\gamma$  by T and NK  
cells, and enhances the lytic activity of NK/LAK cells.  
In addition, IL-12 promotes Th1-type helper cell  
responses. Thus, IL-12 plays a role in cell-mediated  
immunity.

15 IL-12 exerts its biological effects by binding to  
the IL-12 receptor (IL-12R) on the plasma membrane of  
activated T and NK cells (Chizzonite et al., 1992, J.  
Immunol. 148:3117 and Desal et al., 1992, J. Immunol.  
148:3125). IL-12 consists of two subunits referred to as  
20 p40 and p35. The ability of IL-12 to bind to the IL-12R  
has been attributed to the p40 subunit of IL-12. The p35  
subunit, which is linked to the p40 subunit by two  
disulfide bonds, is responsible for signal transduction  
(Gillesse et al., 1995, Eur. J. Immunol. 25:200-206 and  
25 Ling et al., 1995, J. Immunol. 154:116-127).

Summary of the Invention

The invention is based on the discovery that a  
fusion polypeptide that includes an IL-12 p40 subunit  
polypeptide covalently linked to an enzymatically  
30 inactive polypeptide has an *in vivo* half-life that is  
longer than the half-life of native IL-12 p40  
polypeptide. The fusion polypeptides of the invention  
function as antagonists of the IL-12R, and can be used,

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for example, as immunosuppressive agents, e.g., in treating autoimmune diseases.

Accordingly, the invention features a fusion polypeptide that includes an IL-12 p40 subunit polypeptide covalently linked to an enzymatically inactive polypeptide (also referred to herein as the "half-life-increasing polypeptide"), the fusion polypeptide having a circulating half-life *in vivo* that is increased relative to that of the half-life of the native IL-12 p40 polypeptide. Preferably, the *in vivo* half-life of the fusion polypeptide is at least two or more, preferably ten, times that of the half-life of native IL-12 p40 polypeptide.

In one embodiment, the IL-12 p40 subunit polypeptide includes the complete amino acid sequence of the native IL-12 p40 subunit polypeptide. Included within the invention is a polypeptide dimer that includes two fusion polypeptides, each of which includes an IL-12 p40 subunit polypeptide covalently linked to a half-life-increasing, enzymatically inactive polypeptide. Such a dimer, which can be formed spontaneously in a solution of IL-12 p40 fusion polypeptide monomers, can be a heterodimer or, preferably, a homodimer.

The invention also features a nucleic acid encoding the fusion polypeptide that includes an IL-12 p40 subunit polypeptide covalently linked to an enzymatically inactive polypeptide, the fusion polypeptide having a circulating half-life *in vivo* that is increased relative to that of the half-life of native IL-12 p40 polypeptide. Those skilled in the art also recognize that, because of degeneracy in the amino acid code, more than one nucleic acid sequence encodes the IL-12 p40 subunit fusion polypeptides of the invention. All of these nucleic acids, as well as nucleic acids encoding

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the variants described above, are included within the invention.

Preferably, the enzymatically inactive polypeptide of the fusion polypeptide includes a portion of IgG, e.g., the hinge region or the entire the Fc portion of IgG. The Fc portion can include one or more mutations that inhibit complement fixation and/or prevent Fc from binding the Fc receptor with high affinity, thus preventing the fusion polypeptide from being lytic. Alternatively, the Fc portion can be lytic, i.e., able to bind complement and bring about lysis of the cell to which the fusion polypeptide binds. A lytic fusion polypeptide provides a means for effecting lysis of a cell bearing an IL-12 receptor. Such lysis would be desirable, for example, to achieve immunosuppression (e.g., in rescuing a graft from rejection). A non-lytic fusion polypeptide provides a tolerizing therapeutic or immunoprophylactic effect.

Other useful enzymatically inactive polypeptides include proteins that are not enzymes, such as albumin, and enzymes that have enzymatic activity in an organism other than humans but that are inactive in humans. For example, useful polypeptides include plant enzymes, porcine or rodent glycosyltransferases, and  $\alpha$ -1,3-galactosyltransferases (see, e.g., Sandrin et al., 1993, Proc. Natl. Acad. Sci. 90:11391).

The enzymatically inactive polypeptide can include an IgG hinge region and a half-life-increasing enzymatically inactive polypeptide. In this embodiment, the IgG hinge region is covalently linked to the IL-12 p40 subunit polypeptide, and the hinge region serves as a flexible polypeptide spacer between the IL-12 p40 subunit and the enzymatically inactive polypeptide, e.g., albumin. As is described herein, polypeptides other than the IgG hinge region also can serve as the flexible

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polypeptide spacer. When the enzymatically inactive polypeptide includes an IgG hinge region and the Fc portion of an IgG molecule, it lacks an IgG variable region of a heavy chain so that the binding specificity  
5 conferred by the variable region is lacking in the fusion polypeptide.

The fusion polypeptides of the invention can be used, as monomers or dimers, in a variety of therapeutic methods that are included within the invention.  
10 Generally, administration of the fusion polypeptides of the invention to a patient provides a method for suppressing the immune system of the patient. For example, the invention provides a method for treating an autoimmune disease in a patient by administering to the  
15 patient a therapeutically effective amount of an IL-12 p40 subunit fusion polypeptide. An IL-12 p40 subunit fusion polypeptide, when administered to a patient in a therapeutically effective amount, can also be used in a method for inhibiting rejection of a graft in a patient.

20 The invention also includes a method for treating or preventing endotoxin-induced shock in a patient by administering to the patient a therapeutically effective amount of a fusion polypeptide of the invention. In addition, the invention includes a therapeutic  
25 composition that includes (i) a pharmaceutically acceptable carrier and (ii) the above-described fusion polypeptide in which an IL-12 p40 subunit polypeptide and an enzymatically inactive polypeptide are covalently linked, and the resulting fusion polypeptide has a half-  
30 life *in vivo* that is increased relative to that of the half-life of native IL-12 p40 protein.

By "native IL-12 p40 subunit" polypeptide is meant all or a portion of the 40 kD polypeptide described, for exampl , by Gubler et al. (1991, Proc. Natl. Acad. Sci.

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USA 88: 4143-4147; GenBank Accession No. M38444). Gubler et al. describe human IL-12 p40 subunit polypeptide. Also included in the invention is a fusion polypeptide composed of mouse IL-12 p40 subunit; such polypeptides  
5 can be employed in studies involving murine model systems (Gillesen et al., 1995, J. Immunol. 25: 200-206).

In addition, the invention includes fusion polypeptides that include variants of the native IL-12 p40 subunit covalently linked to an enzymatically  
10 inactive polypeptide, as described above. The variants that are suitable for use in the invention are those IL-12 p40 subunit polypeptides that bind the IL-12R in conventional assays. Generally, a fusion polypeptide in which the IL-12 p40 subunit polypeptide has one or a few  
15 amino acid substitutions, or one or a few amino acid deletions, will bind the IL-12R. Variants of the IL-12 p40 subunit polypeptide having a higher or lower affinity for the IL-12R relative to native IL-12 p40 subunit polypeptide also can be used in the invention.

20 By "native" IL-12 protein is meant the full-length, naturally-occurring heterodimeric cytokine, encompassing the p35 and p40 subunits.

By IgG "Fc" portion is meant a naturally-occurring or synthetic polypeptide homologous to the C-terminal  
25 domain of IgG that begins at Proline 238, as defined by Burton et al., 1985, Mol. Immunol. 22:161-206. IgG Fc has a molecular weight of approximately 50 kD. In the molecules of the invention, the entire Fc portion can be used, or only a "half-life-enhancing portion," can be  
30 used. A standard ELISA assay using standard materials and techniques, as described below, can be used to determine whether a portion of the Fc molecule is a "half-life enhancing portion." In addition, many modifications in amino acid sequence are acceptable, as  
35 native activity is not in all cases necessary or desired.

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By "non-lytic" IgG Fc is meant an IgG Fc portion that lacks a high affinity Fc receptor binding site and/or a C'1q binding site. The high affinity Fc receptor binding site includes the Leu residue at position 235 of IgG Fc; the Fc receptor binding site can be functionally destroyed by mutating or deleting Leu 235. For example, substitution of Glu for Leu 235 inhibits the ability of the Fc portion to bind the high affinity Fc receptor (Duncan et al., 1988, Nature 332:563-564). The C'1q binding site can be functionally destroyed by mutating or deleting the Glu 318, Lys 320, and Lys 322 residues of IgG1 (Duncan et al., 1988, Nature 332:738-740). For example, substitution of Ala residues for Glu 318, Lys 320, and Lys 322 renders IgG1 Fc unable to direct Antibody Dependent Cellular Cytotoxicity (ADCC).

By "lytic" IgG Fc is meant an IgG Fc portion that has a high affinity Fc receptor binding site and a C'1q binding site. The high affinity Fc receptor binding site includes the Leu residue at position 235 of the IgG Fc. The C'1q binding site includes the Glu 318, Lys 320, and Lys 322 residues of IgG1. Lytic IgG Fc has wild-type residues or conservative amino acid substitutions at these binding sites. Lytic IgG Fc can target cells for ADCC and/or complement directed cytolysis (CDC).

By IgG "hinge" region is meant a polypeptide homologous to the portion of a naturally-occurring IgG that includes the cysteine residues at which the disulfide bonds linking the two heavy chains of the immunoglobulin form. For IgG1, the hinge region also includes the cysteine residues at which the disulfide bonds linking the  $\gamma$ 1 and light chains form. The hinge region is approximately 13-18 amino acids in length in IgG1, IgG2, and IgG4, and, approximately 65 amino acids in length in IgG3.



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By "polypeptide spacer" is meant a polypeptide that, when placed between an IL-12 p40 subunit polypeptide and the enzymatically inactive, half-life-increasing polypeptide, possesses an amino acid residue  
5 with a normalized B value ( $B_{\text{norm}}$ ; a measure of flexibility) of 1.000 or greater, preferably of 1.125 or greater, and, most preferably of 1.135 or greater (see, e.g., Karplus et al., 1985, Naturwissenschaften 72:212). Amino acids that are commonly known to be flexible  
10 include glutamic acid, glutamine, threonine, lysine, serine, glycine, proline, aspartic acid, asparagine, and arginine.

The invention offers several features and advantages: (1) the fusion polypeptides of the invention  
15 have an extended circulating half-life and provide long term protection; (2) because the IL-12 p40 subunit and many of the enzymatically inactive polypeptides useful in the invention have already been purified, the fusion polypeptides can easily be purified by employing methods  
20 that have been described for purifying the IL-12 p40 subunit or for purifying the enzymatically inactive polypeptides; (3) in certain embodiments, the fusion polypeptide is mutated such that it is defective for ADCC and CDC, thus making the fusion polypeptide useful for  
25 treating or preventing diseases, such as autoimmune diseases, without destroying the target cells; and (4) in certain embodiments, the fusion polypeptide includes, as the enzymatically inactive protein, the Fc portion of IgG; when Fc is included, the preferred, dimeric fusion  
30 polypeptides of the invention can be purified in one step with affinity chromatography employing protein A.

An additional advantage of fusion polypeptides that include an Fc polypeptide is that they cannot cross the blood/brain barrier into the brain where IL-12 is  
35 thought to cause undesirable side effects, e.g.,

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somnolence, fever, and hypotension, by reacting with regulatory centers in the brain.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

## Detailed Description

The drawings will first be briefly described.

### Drawings

Fig. 1 is a schematic representation of human IL-12 p40/Fcγ1, one of the fusion polypeptides of the invention. For comparison, various immunoglobulins also are shown.

Fig. 2 is a schematic representation of the synthetic oligonucleotides and cDNAs used to fuse DNA encoding the IL-12 p40 subunit to DNA encoding Fcγ1 (SEQ ID NOS:1-16). Asterisks indicate that the sequences shown are the inverse conjugates of the actual oligonucleotide sequences. "cDNA transl" indicates the amino acid sequence obtained from the native cDNA sequence. "PCR transl" indicates the amino acid

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translation of the PCR product. "Ter" represents a termination codon. Differences between the PCR product and the native cDNA are underlined. The beginning of the Fc sequence is enclosed in the rectangle in the final fusion junction (SEQ ID NOS:15 and 16).

Fig. 3 is a map of the plasmid h12p40/G1/Bam<sup>-</sup>3/18, an intermediate plasmid used to produce a final plasmid that expresses the IL-12 p40/Fcγ1 fusion protein. Plasmid h12p40/G1/Bam<sup>-</sup>3/18 includes a cytomegalovirus promoter-enhancer element located 5' to the site at which the fusion cDNA was inserted. The plasmid also includes a polyadenylation signal and transcription termination sequences from bovine growth hormone, and neomycin and ampicillin resistance genes. The fusion cDNA insert occupies nucleotides 923-2665, with the sequence between nucleotides 923 and 962 having been carried over from the TA cloning vector.

Fig. 4 is a map of the plasmid p40/γ1 3/31, the final expression plasmid bearing the human IL-12 p40/Fcγ1 gene fusion. The fusion gene insert occupies nucleotides 896-2592. The flanking *Hind*III and *Xba*I sites are at positions 890 and 2593, respectively. The plasmid lacks the TA cloning vector sequences present in h12p40/G1/Bam<sup>-</sup>3/18. The junction between the IL-12 p40 sequence and the Fc sequence spans the unique *Bam*HI site at position 1892.

#### IL-12 p40 Subunit Fusion Polypeptides

Conventional molecular biology techniques can be used to produce fusion polypeptides having the IL-12 p40 subunit polypeptide covalently linked to an enzymatically inactive polypeptide, e.g., a lytic or non-lytic Fc portion of IgG. Numerous polypeptides are suitable for use as enzymatically inactive polypeptides in the invention. Preferably, the enzymatically inactive

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polypeptide has a molecular weight of at least 10 kD, a net neutral charge at pH 6.8, a globular tertiary structure, human origin, and no ability to bind to surface receptors other than the IL-12 receptor.

5           Where the enzymatically inactive polypeptide is Fc, the IgG portion preferably is glycosylated. If desired, the enzymatically inactive polypeptide can include an IgG hinge region positioned such that the fusion polypeptide has an IL-12 p40 subunit polypeptide  
10 bonded to an IgG hinge region with the hinge region bonded to a half-life-increasing polypeptide. Thus, the hinge region can serve as a spacer between the IL-12 p40 subunit polypeptide and the half-life-increasing polypeptide. A person skilled in molecular biology can  
15 readily produce such molecules in eukaryotic cells or baculovirus systems, for example. As an alternative to using an IgG hinge region, a flexible polypeptide spacer, as defined herein, can be used. Using conventional molecular biology techniques, such a polypeptide can be  
20 inserted between the IL-12 p40 subunit polypeptide and the half-life increasing polypeptide.

          Where the enzymatically inactive polypeptide includes an Fc portion, the Fc portion can be mutated, if desired, to inhibit its ability to fix complement and/or  
25 bind the Fc receptor with high affinity. For example, for murine IgG Fc, substitution of Ala residues for Glu 318, Lys 320, and Lys 322 renders the polypeptide unable to direct CDC. Substitution of Glu for Leu 235 inhibits the ability of the polypeptide to bind the Fc receptor  
30 with high affinity. Appropriate mutations for human IgG also are known (see, e.g., Morrison et al., 1994, The Immunologist 2: 119-124 and Brekke et al., 1994, The Immunologist 2: 125). Other mutations can also be used to inhibit these activities of the polypeptide, and art-  
35 recognized methods can be used to assay for the ability

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of the polypeptide to fix complement or bind the Fc receptor.

Other useful enzymatically inactive polypeptides include albumin, e.g., human serum albumin, transferrin, 5 enzymes such as t-PA that have been inactivated by mutations, and other polypeptides having a long circulating half-life and without enzymatic activity in humans.

Preferably, the enzymatically inactive 10 polypeptide, e.g., IgG Fc used in the production of the fusion polypeptide has, by itself, an *in vivo* circulating half-life greater than that of the native IL-12 p40 subunit, so that the *in vivo* circulating half-life of the fusion polypeptide is greater than that of the native IL-15 12 p40 subunit. More preferably, the half-life of the fusion polypeptide is at least 2 times that of native IL-12 p40 subunit alone. Most preferably, the half-life of the fusion polypeptide is at least 10 times that of native IL-12 p40 subunit alone. The circulating half-20 life of the fusion polypeptide can be measured in an ELISA of a sample of serum obtained from a patient treated with the fusion polypeptide. In such an ELISA, antibodies directed against the IL-12 p40 subunit polypeptide can be used as the capture antibodies, and 25 antibodies directed against the enzymatically inactive polypeptide can be used as the detection antibodies, allowing detection of only the fusion polypeptide in a sample (see, e.g., Ling et al., 1995, J. Immunol. 154:116-127). Conventional methods for generating 30 antibodies and performing ELISAs can be used for all assays described herein.

The fusion polypeptides can be synthesized using conventional methods for protein expression using recombinant DNA technology. Because the IL-12 p40 35 subunit polypeptide has been purified previously, many of

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the previously-described methods for protein purification are useful, alone or along with other conventional methods, for purifying the fusion polypeptides of the invention (see, e.g., Gillessen et al., 1995, J. Immunol. 5 25:200-206). If desired, the fusion polypeptide can be affinity-purified according to standard protocols with antibodies directed against the IL-12 p40 subunit. Antibodies directed against the enzymatically inactive polypeptide also are useful for purifying the fusion  
10 polypeptide by conventional immunoaffinity techniques. Fusion polypeptides that include Fc can be purified using Protein A column chromatography. If desired, the function of the fusion polypeptide can be assayed with methods that are commonly used to test the function of  
15 the IL-12 p40 subunit alone. It is not necessary that the fusion polypeptide bind the IL-12R in a manner identical to that in which the native IL-12 p40 subunit binds the IL-12R. For example, the fusion polypeptide can bind the IL-12R more or less strongly than does the  
20 native IL-12 p40 subunit.

Example: Construction of an IL-12 p40  
Subunit Polypeptide/Fcγ1 Fusion Polypeptide

To produce a fusion polypeptide that includes a human IL-12 (hIL-12) p40 subunit and human Fcγ1, the cDNA  
25 for the hIL-12 p40 subunit was isolated and ligated into an expression plasmid along with the sequence encoding human Fcγ1. The resulting fusion polypeptide is shown schematically in Fig. 1.

Human cDNA was used as a template for DNA  
30 synthesis in a polymerase chain reaction (PCR). The synthetic oligonucleotide primers and cDNA templates are presented schematically in Fig. 2. The following synthetic oligonucleotide primers were produced and purified according to conventional protocols.

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Oligonucleotide #3441, an hIL-12 p40 antisense oligonucleotide, has the sequence:

5'-GGATCCGAGCAGGGCACAGATGCCCATTTCGC-3' (SEQ ID NO:4).

This oligonucleotide places a unique *Bam*HI site at the 3' end of the sequence encoding the IL-12 p40 subunit polypeptide, and changes the codon of the terminal Serine residue from AGT to TCG. Oligonucleotide #3442, an hIL-12 p40 sense oligonucleotide, has the sequence:

5'-AAGCTTGGCCCAGAGCAAGATGTGTCACC-3' (SEQ ID NO:1); this oligonucleotide places a *Hind*III site at the 5' end of the sequence encoding the IL-12 p40 subunit polypeptide.

The conditions for PCR amplification of the DNA encoding the hIL-12 p40 subunit polypeptide were as follows:

15	5.0 $\mu$ l 10X Mg <sup>+2</sup> reaction buffer
	5.0 $\mu$ l 1 mM dNTPs
	1.0 $\mu$ l sense oligonucleotide (#3442)
	1.0 $\mu$ l antisense oligonucleotide (#3441)
	1.5 $\mu$ l human cDNA
20	36 $\mu$ l water
	0.5 $\mu$ l Taq polymerase

Thirty-five cycles of synthesis were carried out, with each cycle including 1 minute of incubation at each of 94°C, 55°C, and then 72°C. Agarose gel electrophoresis of the synthesized DNA revealed a 1 kbp product, which, based on the sequence of hIL-12, is the predicted size of the amplified fragment. The identity of the amplified fragment was further verified by *Eco*RI restriction analysis, with the enzyme producing fragments of 750 bp and 250 bp, as is desired.

The synthesized hIL-12 p40 subunit DNA was then ligated into a TA cloning vector, pCRII (Invitrogen, San Diego, CA). Recombinant plasmids were obtained by transforming competent DH5 $\alpha$  *E. coli* (BRL/Gibco, Grand Island, NY) with the ligation product. Clones having the desired DNA inserted in the correct orientation were

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identified by restriction enzyme analysis using the enzymes *EcoRI*, *NotI*, and *BamHI*. The hIL-12 p40 cDNA was then isolated by digesting the desired recombinant plasmid with *NotI* and *BamHI*, and isolating the fragment of approximately 1,000 bp (e.g., from a low-melt agarose gel). This fragment of hIL-12 p40 cDNA was then ligated to Fc $\gamma$ 1 cDNA produced as described below.

The cDNA encoding human Fc $\gamma$ 1 was isolated from human cDNA by employing synthetic oligonucleotides #580 and #3464. The sense oligonucleotide, #580, has the sequence 5'-CCTGACGGATCCCAAATCTGCTGACAAAACCTCACACATGCCCA-3' (SEQ ID NO:8). The antisense Fc $\gamma$ 1 oligonucleotide, #3464, has the sequence 5'-GCTCTAGACTCATTACCCGGAGACAGGG-3' (SEQ ID NO:12). Oligonucleotide #580 changes the first codon of the hinge region of Fc $\gamma$ 1 from GAG (encoding Glutamic acid) to GAT (encoding Aspartic Acid), creating a unique *BamHI* restriction site at the 5' end of the resulting PCR product. This oligonucleotide also changes the codon for the fifth amino acid from TGT (encoding Cysteine) to GCT (encoding Alanine). This Cysteine residue normally participates in forming a disulfide bridge between the heavy and light chains of the immunoglobulin. Mutation of the Cysteine residue prevents undesired pairing; the Alanine substitution nonetheless retains maximal flexibility in the hinge region.

Oligonucleotide #3464 adds a unique *XbaI* site to the 3' end of the sequence encoding Fc $\gamma$ 1 (Fig. 2). For the sequence of human IgG, see, e.g., Takahashi et al., 1982, Cell 29: 671-679 and Ellison et al., 1982, Nucl. Acids Res. 10:4071-4079; GenBank Accession No. J00228. The DNA encoding Fc $\gamma$ 1 was amplified by PCR using DNA encoding synthetic IL-2/Fc $\gamma$ 1 (sIL-2/Fc $\gamma$ 1) as a template. The PCR reaction mixture contained

5.0  $\mu$ l 10X Mg<sup>+2</sup> reaction buffer



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5.0  $\mu$ l 1 mM dNTPs  
1.0  $\mu$ l sense oligonucleotide (#580)  
1.0  $\mu$ l antisense oligonucleotide (#3464)  
1.5  $\mu$ l sIL-2/Fc $\gamma$ 1 as a DNA template  
5 36  $\mu$ l water  
0.5  $\mu$ l Taq polymerase

Synthesis was allowed to proceed for 35 cycles, with each cycle including incubation at 94°C for 60 seconds, 53°C for 45 seconds, and 72°C for 45 seconds. Production of  
10 the desired 700 bp product was confirmed by subjecting an aliquot of the PCR product to agarose gel electrophoresis. The amplified DNA encoding Fc $\gamma$ 1 then was ligated into a TA vector, and competent DH5 $\alpha$  *E. coli* were transformed with the ligation product. The desired  
15 clones were identified by restriction enzyme analysis using *Eco*RI, *Bam*HI, and *Xba*I. To isolate DNA encoding the Fc $\gamma$ 1 fragment, DNA of a desired clone was digested with *Bam*HI and *Xba*I, and the liberated 700 bp product was purified from a low-melt agarose gel.

20 The 700 bp Fc $\gamma$ 1 DNA and the hIL-12 p40 DNA were co-ligated into a modified version of the eukaryotic expression vector pRc/CMV (Invitrogen). This expression vector was first modified to remove all of the *Bam*HI sites. The three-way ligation mixture containing the  
25 following components was incubated overnight at 16°C.

1  $\mu$ l pRc/CMV (*Not*I/*Xba*I ends)  
1  $\mu$ l hIL-12 p40 (*Not*I/*Bam*HI ends)  
1  $\mu$ l Fc $\gamma$ 1 (*Bam*HI/*Xba*I ends)  
4  $\mu$ l 5X ligase buffer  
30 12  $\mu$ l water  
1  $\mu$ l T4 DNA ligase

After transforming competent DH5 $\alpha$  *E. coli* with the ligation mixture, the desired clones were identified by restriction enzyme analysis with *Pst*I and *Xma*I,  
35 separately and in combination. This plasmid, h12p40/G1/*Bam*<sup>-</sup>3/18, is shown schematically in Fig. 3.

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Digestion of the plasmid with *NotI* and *BamHI* confirmed that the IL-12 p40 sequence was intact, and digestion with *BamHI* and *XbaI* confirmed that the Fc $\gamma$ 1 sequence was intact.

5 In performing the cloning strategy outlined above, a portion of the TA vector (nucleotides 923-962 of the plasmid) was inadvertently included in the plasmid. To correct this, the entire sequence encoding the hIL-12 p40/Fc $\gamma$ 1 fusion polypeptide was excised with *HindIII* and  
10 *XbaI*, and ligated into a second modified pRc/CMV expression plasmid that had been digested with *HindIII* and *XbaI*. This second modified pRc/CMV plasmid was constructed by substituting the *BglIII-XbaI* sequence of the original pRc/CMV vector for the corresponding  
15 sequence in the first modified version of pRc/CMV (i.e., the plasmid that lacked *BamHI* sites). This cloning step reintroduced into the multiple cloning site a unique *HindIII* site that had been removed in producing the first modified version of pRc/CMV. This final plasmid, termed  
20 "p40/ $\gamma$ 1 3/31" has a unique *BamHI* site at the junction between the sequences encoding the IL-12 p40 subunit polypeptide and Fc $\gamma$ 1 (Fig. 4). As is desired, this plasmid lacks sequences from the TA cloning vector, and it has unique *HindIII* and *XbaI* sites flanking the  
25 inserted fusion gene. The fusion polypeptide can be expressed by transfecting the expression plasmid p40/ $\gamma$ 1 3/31 into cultured eukaryotic host cells. The fusion polypeptide can be secreted into, and purified from, the cell culture medium.

### 30 Therapeutic Use of IL-12 p40 Subunit Fusion Polypeptides

A therapeutic composition that includes a fusion polypeptide(s) of the invention can be formulated, according to standard protocols, by admixture of the fusion polypeptide and a pharmaceutically acceptable

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carrier such as water or saline. If desired, a combination of fusion polypeptides, e.g., IL-12 p40 subunit linked to Fc $\gamma$ 1 and IL-12 p40 subunit linked to albumin, can be administered to a patient, either  
5 sequentially or simultaneously. The therapeutic composition can include monomers or dimers of the fusion polypeptides of the invention; in addition, a mixture of monomers and dimers can be used. Preferably, a substantial number of the fusion polypeptides of the  
10 therapeutic composition are in the dimeric form. In practicing the invention, however, it is not necessary to analyze the therapeutic composition for its content of dimers and monomers.

The fusion polypeptide can be administered to a  
15 patient intravenously, intraperitoneally, intramuscularly, and/or subcutaneously. Generally, a fusion polypeptide dosage of 1  $\mu$ g/kg body weight to 500 mg/kg body weight can be used; preferably, the dosage is 10  $\mu$ g/kg body weight to 100  $\mu$ g/kg body weight.  
20 Preferably, the fusion polypeptide is administered before or at the first sign of disease onset; if desired, the fusion polypeptide can be administered before signs of disease appear. Those skilled in the art of medicine will be able to adjust the dosage and frequency of  
25 administration as desired. Generally, the fusion polypeptides will be administered at regular, e.g., 12-hour, intervals. The efficacy of the treatment can be determined by monitoring the patient for commonly-known signs of the disease, or by assaying fluid (e.g., serum)  
30 samples of the patient for the presence of the fusion polypeptide.

If desired, the therapeutic value of a fusion polypeptide of the invention can be estimated in an in vivo model system of disease. For example, one can use a  
35 murine model of endotoxin-induced shock to demonstrate

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that an IL-12 p40 subunit fusion polypeptide acts as an IL-12R antagonist and inhibits IL-12-induced production of interferon- $\gamma$  (see Wysocka et al., 1995, Eur. J. Immun. 25:672-676). Mice that are primed with the avirulent  
5 Bacille Calmette Guerin (BCG) vaccine strain of *Mycobacterium bovis* and treated with endotoxins of Gram-negative bacteria (lipopolysaccharides; LPS) produce IL-12, which controls the production of interferon- $\gamma$ . Neutralizing anti-IL-12 antibodies inhibit LPS-induced  
10 production of interferon- $\gamma$ , and completely protect BCG-primed mice from the lethal effects of LPS. Thus, IL-12 is required for interferon- $\gamma$  production and lethality in a model of endotoxin-induced shock in mice. IL-12 p40 subunit fusion polypeptides that antagonize IL-12R in  
15 this assay have the potential to treat or prevent endotoxin-induced shock in humans.

The therapeutic value of the fusion polypeptides of the invention can also be estimated in mice that have experimental allergic encephalomyelitis (EAE; see Leonard  
20 et al., 1995, J. Exp. Med. 181:381-386). EAE can be induced by transferring into naive mice lymph node cells that are (a) isolated from mice primed with proteolipid protein (PLP) and (b) stimulated *in vitro* with PLP. When recombinant murine IL-12 is added to the *in vitro*  
25 stimulation reaction, the resulting course of disease is more severe. Thus, the therapeutic value of the fusion polypeptides of the invention can be demonstrated by measuring their ability to diminish to IL-12 enhancement of EAE.

30 In another method, the therapeutic value of the fusion polypeptides of the invention can be assessed by measuring their ability to inhibit IL-12-induced autoimmune diabetes in NOD mice (see Trembleau et al., 1995, J. Exp. Med. 181:817-821). IL-12 p40 subunit  
35 fusion polypeptides that inhibit IL-12-induced autoimmune

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diabetes in NOD mice are potential diabetes therapeutics.

In an alternative assay, the therapeutic value of the fusion polypeptides can be estimated by measuring their ability to inhibit IL-12-mediated islet allograft rejection in mice (see Gish et al., 1995, Transpl. Proc. 27:459-460). Fusion polypeptides that inhibit allograft rejection in this assay are potential immunosuppressive agents. These examples are meant to be illustrative, not limiting; other art-recognized assays also can be performed to estimate the therapeutic value of the IL-12 p40 subunit fusion polypeptides of the invention. In addition, the IL-12 p40 subunit fusion polypeptides of the invention have potential therapeutic value in treating any disorder that is exacerbated by IL-12.

15 Determination of the Circulating  
Half-life of a Fusion Polypeptide

To measure the circulating half-life of a fusion polypeptide of the invention, the serum concentration of the fusion polypeptide can be determined over time following a single bolus intravenous injection of the fusion polypeptide into 8- to 10- week old BALB/c mice (Jackson Laboratory). Serial 100  $\mu$ l blood samples can be obtained by retro-orbital bleeding over time, e.g., at 0.1, 6, 24, 48, 72, and 96 hours after administration of the fusion polypeptide to the mice. Measurements of the circulating half-life can be made, for example, in an ELISA with a rat-anti-mouse IL-12 p40 mAb as the capture antibody, and a horseradish peroxidase conjugated rat-anti-mouse Fcy2a monoclonal antibody as the detection antibody (PharMingen), thus assuring that this assay is specific for the IL-12 p40/Fc fusion polypeptide and not the IL-12 p40 subunit alone or mIgG2a alone.

Such an assay demonstrates that IL-12 p40/Fc possesses a prolonged circulating half-life. Attenuation

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of the FcγRI binding abilities due to the specific mutations introduced into the Fcγ2a CH2 domain can also be measured. In addition, the ability of the mutation in the C'1q binding site to diminish the ability of the Fcγ2a domain to activate complement can be determined in a complement lysis assay. Such an assay would involve measuring the release of <sup>51</sup>Cr from <sup>51</sup>Cr-labeled targeted cells; mutant fusion polypeptides would not induce lysis, while wild-type fusion polypeptides would induce lysis and release of <sup>51</sup>Cr. These methods allow one to determine if the ability of IL-12 p40/Fc to support CDC has been eliminated.

#### Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

For example, any mutation that results in the disabling of the complement-fixing and/or high-affinity binding capability of the Fc portion of an antibody is within the scope of the invention.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: University of Massachusetts  
Beth Israel Hospital
- (ii) TITLE OF INVENTION: IL-12 FUSION POLYPEPTIDES AND  
USES THEREOF
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson P.C.
  - (B) STREET: 225 Franklin Street
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/565,856
  - (B) FILING DATE: 01 DEC 1995
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: J. Peter Fasse
  - (B) REGISTRATION NUMBER: 32,983
  - (C) REFERENCE/DOCKET NUMBER: 04020/079WO1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617/542-5070
  - (B) TELEFAX: 617/542-8906
  - (C) TELEX: 200154

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AAGCTTGGCC CAGAGCAAGA TGTGTCACC

29

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs

- 22 -

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGGCCCCAGAG CAAGATGTGT CACC

24

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 3 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Cys His

1

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGAATGGGC ATCTGTGCCC TGCTCGGATC C

31

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCGAATGGGC ATCTGTGCCC TGCAGTTAG

29

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 8 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Glu Trp Ala Ser Val Pro Cys Ser  
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 9 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Trp Ala Ser Val Pro Cys Ser Asp  
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 43 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTGACGGAT CCCAAATCTG CTGACAAAAC TCACACATGC CCA

43

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAGCCCAAAT CTTGTGACAA AACTCACACA TGCCCA

36

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 12 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro  
1 5 10

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## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Asp Pro Lys Ser Ala Asp Lys Thr His Thr Cys Pro  
1 5 10

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCCTGTCTCC GGGTAAATGA GTCTAGAGC

29

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCTGTCTCC GGGTAAATGA

20

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Leu Ser Pro Gly Lys  
1 5

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 62 base pairs
  - (B) TYPE: nucleic acid

- 25 -

(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCGAATGGGC ATCTGTGCCC TGCTCGGATC CCAAATCTGC TGACAAAAC 50  
CACACATGCC CA 62

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Glu Trp Ala Ser Val Pro Cys Ser Asp Pro Lys Ser Ala Asp 1  
5 10  
Lys Thr His Thr Cys Pro  
15 20

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What is claimed is:

1. A fusion polypeptide comprising an IL-12 p40 subunit polypeptide and an enzymatically inactive polypeptide covalently linked to said IL-12 p40 subunit polypeptide, said fusion polypeptide having a circulating half-life *in vivo* that is longer than increased relative to that of the half-life of native IL-12 p40 subunit protein.
2. A fusion polypeptide according to claim 1, wherein said IL-12 p40 subunit polypeptide consists of the complete amino acid sequence of native IL-12 p40 subunit polypeptide.
3. An IL-12 p40 subunit fusion polypeptide dimer comprising two fusion polypeptides of claim 1.
4. An IL-12 p40 subunit fusion polypeptide dimer according claim 3, wherein said p40 subunit polypeptide consists of the complete amino acid sequence of the native IL-12 p40 subunit polypeptide.
5. A fusion polypeptide of claim 1 or 3, for use in treating an autoimmune disease.
6. A fusion polypeptide of claim 1 or 3, for use in inhibiting rejection of a graft.
7. A fusion polypeptide of claim 1 or 3, for use in treating or preventing endotoxin-induced shock.
8. A nucleic acid encoding the fusion polypeptide of claim 1.

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9. A fusion polypeptide of claim 1, wherein said enzymatically inactive polypeptide comprises a portion of IgG.

10. A fusion polypeptide according to claim 9,  
5 wherein said portion of IgG is Fc.

11. A fusion polypeptide of claim 1, wherein said enzymatically inactive polypeptide comprises an IgG hinge region.

12. A fusion polypeptide of claim 1, wherein said  
10 enzymatically inactive polypeptide comprises albumin.

13. A fusion polypeptide of claim 1, wherein said enzymatically inactive polypeptide lacks an IgG variable region of a heavy chain.

14. A fusion polypeptide of claim 10, wherein  
15 said Fc portion is lytic.

15. A fusion polypeptide of claim 10, wherein said Fc portion includes a mutation that inhibits complement fixation by said fusion polypeptide.

16. A fusion polypeptide of claim 10, wherein  
20 said Fc portion includes a mutation that inhibits high affinity binding to the Fc receptor by said fusion polypeptide.

17. A fusion polypeptide of claim 10, wherein said Fc portion includes a mutation that inhibits  
25 complement fixation and high affinity binding to the Fc receptor by said fusion polypeptide.

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18. A fusion polypeptide of claim 1, wherein said enzymatically inactive polypeptide comprises a polypeptide spacer.

19. A therapeutic composition comprising a fusion  
5 polypeptide of claim 1 or 3 admixed with a pharmaceutically acceptable carrier.

20. The use of a fusion polypeptide of claim 1 or  
3 for the manufacture of a medicament for treating  
autoimmune disease, graft rejection, or endotoxin-induced  
10 shock.

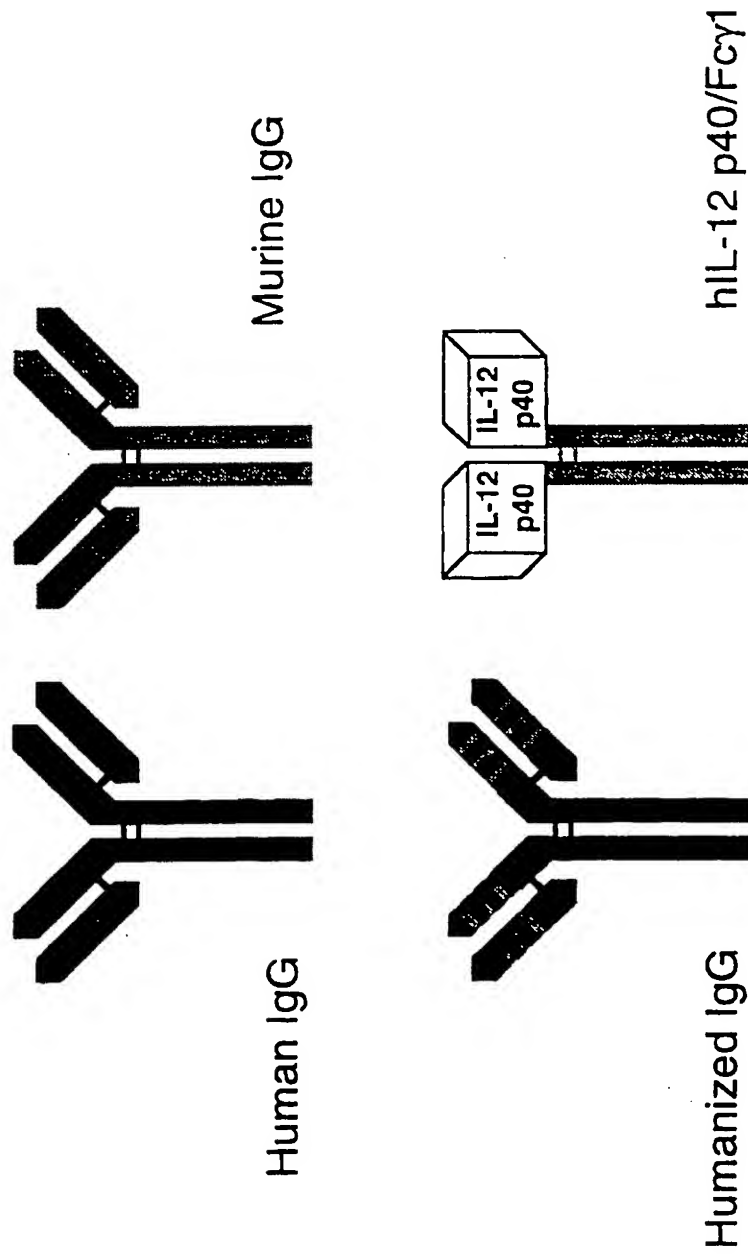


FIG. 1

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## IL-12 p40 cDNA Generation

SEQ ID NO:

3442	5'- <u>AAGCTTGGCCCAGAGCAAGATGTGTCACC</u>	1
cDNA	5'-TGGCCCAGAGCAAGATGTGTCACC...	2
cDNA transl	MetCysHis...	3
PCR transl	MetCysHis...	3
3441*	5'-GCGAATGGGCATCTGTGCCCTGCT <u>CGGATCC</u>	4
cDNA	5'-...GCGAATGGGCATCTGTGCCCTGCAGTTAG	5
cDNA transl	...GluTrpAlaSerValProCysSerTer	6
PCR transl	...GluTrpAlaSerValProCysSer <u>Asp</u> ...	7

## Fcγ1 cDNA Generation

SEQ ID NO:

580	5'- <u>CCTGACGGATCCCAAATCTGCTGACAAA</u> ACTCACACATGCCCA	8
cDNA	5'-GAGCCCAAATCTTGTGACAAAACTCACACATGCCCA...	9
cDNA transl	GluProLysSerCysAspLysThrHisThrCysPro...	10
PCR transl	<u>Asp</u> ProLysSer <u>Ala</u> AspLysThrHisThrCysPro...	11
3464*	5'-CCCTGTCTCCGGGTAAATGAGTCTAGAGC	12
cDNA	5'-...CCCTGTCTCCGGGTAAATGA	13
cDNA transl	...LeuSerProGlyLysTer	14
PCR transl	...LeuSerProGlyLysTer	14

## Final Fusion Junction

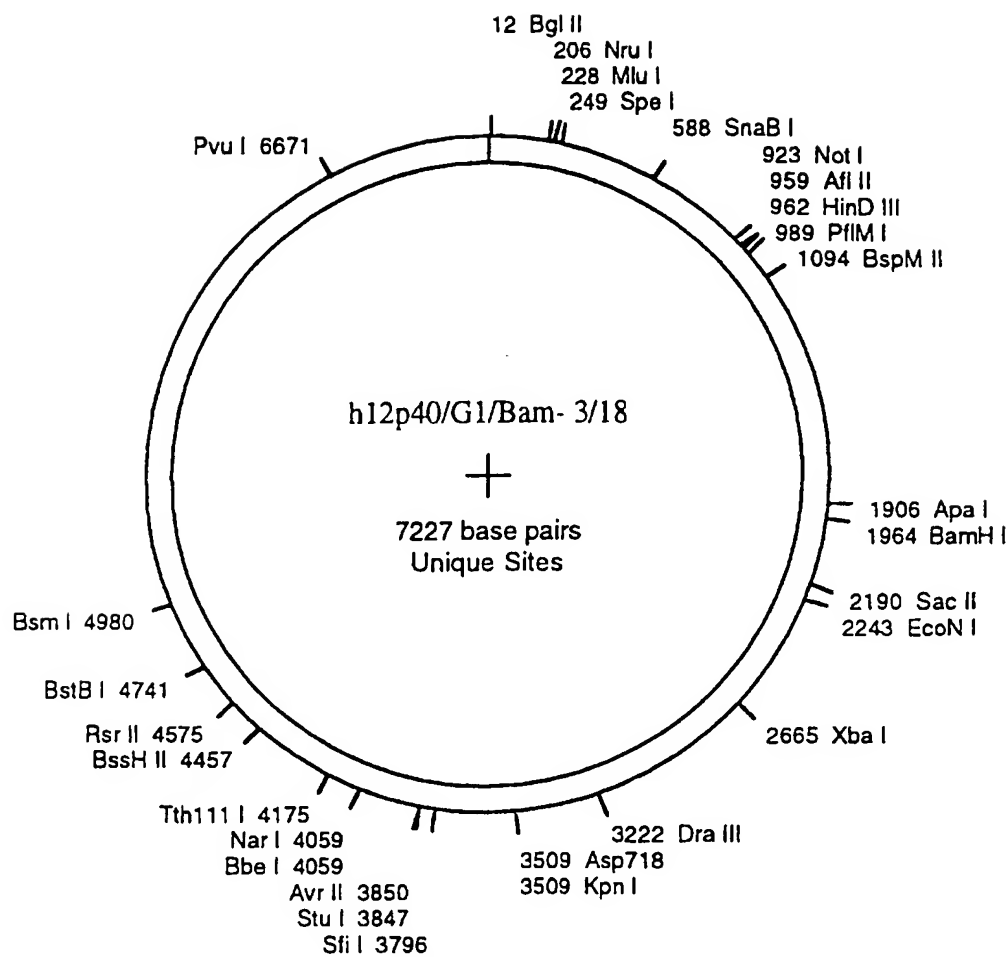
SEQ ID NO:

5'-GCGAATGGGCATCTGTGCCCTGCTCG	<u>GATCCCAAATCTGCTGACAAA</u> ACTCACACATGCCCA	15
...GluTrpAlaSerValProCysSer	<u>Asp</u> ProLysSer <u>Ala</u> AspLysThrHisThrCysPro	16

FIG. 2



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**FIG. 3**

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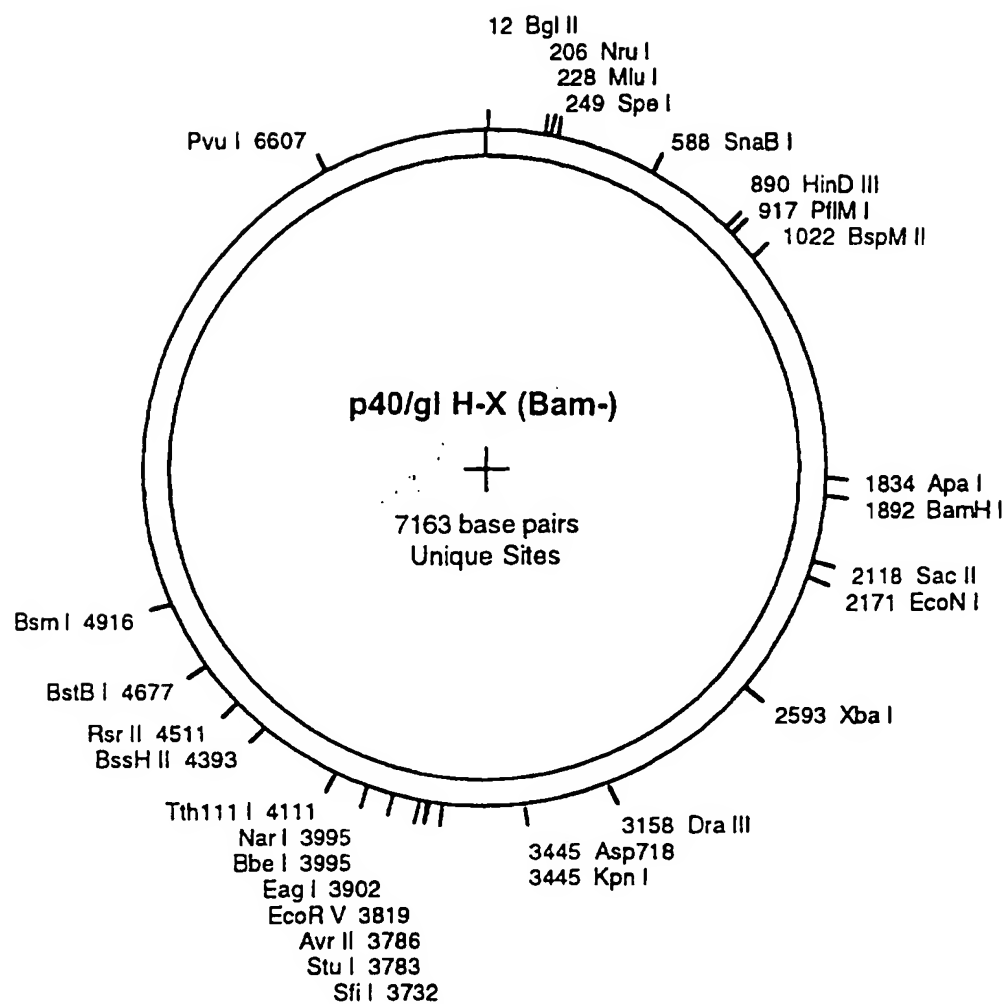


FIG. 4